Pest Management Grants Final Report
98-0273

Development of Alternative Strategies for Chemical Control of Pre- and Post-harvest Brown Rot of Stone Fruits

Themis J. Michailides
University of California Davis, Department of Plant Pathology,
Kearney Agricultural Center, Parlier, CA 93648
Phone 559-646-6546; Fax 559-646-6593
Themis@uckac.edu

Cooperating Personnel: Drs. C. Hong, Y. Luo, and E. Boehm
D. Morgan (SRA), and D. Felts (Lab. Assistant)

31 March 2000

Prepared for California Department of Pesticide Regulation

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Acknowledgments

We thank the California Department of Pesticide Regulation for financial support of this project. In addition, we thank the California Tree Fruit Agreement, the California Prune Board, and a University of California BioSTAR project for partial support of this project.

This report was submitted in fulfillment of **DPR98-0273 Development of Alternative Strategies for Chemical Control of Pre- and Post-harvest Brown Rot of Stone Fruits** project by the University of California under the partial sponsorship of the California Department of Pesticide Regulation. Work was completed as of March 31, 2000.

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Abstract

The general scope of this study was to study alternative strategies for chemical control of pre- and post-harvest brown rot of stone fruits using traditional as well as more advanced state of the art molecular procedures. Because of the unfavorable conditions for development of latent infection by *Monilinia fructicola* we were not able to compare the applied ONFIT technique with the species-specific CPR primers or the genomic clones that we have developed for the major brown rot pathogen, *Monilinia fructicola*. However, we were able to set up a state of the art, fully equipped molecular laboratory at the Kearney Agricultural Center and to develop two very specific PCR primers (Mf2A and Mf2B) which detect DNA of *Monilinia fructicola*. These primers also amplified and detected DNA of *Monilinia fructicola* in stone fruit blossoms, and in inoculated fruit. In addition, we have identified three high copy, genomic clones (pMF73, pMF150, and pMF210) that are specific to a world-wide collection of *M. fructicola* isolates, to the exclusion of other fungi commonly encountered from stone fruit surface, including the closely related *M. laxa.* These high copy clones are sensitive enough to detect between 10-50 pg of fungal DNA in dot blot hybridizations. Analyses of fruit with suspected latent infections of *M. fructicola* collected from 29 orchards in 1998 using the PCR primers and/or the specific genomic clones are in progress. In 1999, we repeated the experiment of spraying with iprodione fruit on the floor that were thinned at a post pit-hardening stage in two orchards. We found significant differences in 1998, but not in 1999. In 1999 due to dry, unfavorable conditions brown rot did not develop in any of the treatments, so comparisons could not be made between the treated and untreated thinned fruit. Biological control experiments using *Trichoderma viride* and its metabolite 6-pentyl-*2H*-pyrna-2-one (6PAP), and an isolate of *Bacillus subtilis* isolated from stone fruits were the most efficacious in inhibiting brown rot development on Casselman plums. In contrast, the commercial ASPIRE™ (a.i. *Candida oleophila,* isolate I-182) and the semi-commercial biofungicide CIM (a.i., *Cryptococcus infirmo-miniatus*) did not reduce brown rot incidence.

Executive Summary

There were three objectives in this study as follows: 1) to determine the relative importance of long- and short-term latent infection for pre- and post-harvest brown rot of stone fruits to help reduce sources of primary and secondary spore inoculum and predict disease at harvest and post-harvest. 2) To evaluate alternative strategies for chemical control such as burying thinned fruit underneath by disking or rototilling, and/or cover-spraying thinned fruit on the ground with a registered fungicide in comparison with the conventional practice of leaving thinned fruit on the orchard floor. And 3) to select biological control agents against post-harvest brown rot of stone fruits. Because of the unfavorable conditions for development of latent infection by *Monilinia fructicola* we were not able to compare the applied ONFIT technique with the species-specific CPR primers or the genomic clones that we have developed for the major brown rot pathogen, *Monilinia fructicola.* However, during 1998 and 1999 we were able to set up a state of the art, fully equipped molecular laboratory at the Kearney Agricultural Center. Two very specific PCR primers, Mf2A and Mf2B, have been developed and can detect DNA of *Monilinia fructicola.* These primers also amplified and detected DNA of *Monilinia fructicola* in stone fruit blossoms,

and in inoculated fruit. In addition, we have identified three high copy, genomic clones (pMF73, pMF150, and pMF210) from a partial Sau3AI genomic library that are specific to a world-wide collection of *M. fructicola* isolates, to the exclusion of other fungi commonly encountered from stone fruit surface, including the closely related *M. laxa.* These high copy clones are sensitive enough to detect between 10-50 pg of fungal DNA in dot blot hybridizations. Analyses of fruit with suspected latent infections of *M. fructicola* collected from 29 orchards in 1998 using the PCR primers and/or the specific genomic clones are in progress. Burying thinned fruit by disking prevented sporulation of *M. fructicola* because almost all the fruits were buried under the ground. This method then can reduce production of secondary inoculum by the pathogen. Spraying thinned fruit with iprodione (Rovral®) reduced sporulation on thinned fruit in a 1998 experiment, thus also reducing secondary spore inoculum. However, in 1999 when this experiment was repeated in two orchards because of unfavorable conditions brown rot did not develop in any of the treatments, and we could not compare the treatments. Biological control experiments using *Trichoderma viride* and its metabolite 6-pentyl-*2H*-pyrna-2-one (6PAP), and an isolate of *Bacillus subtilis* isolated from stone fruits were the most efficacious in inhibiting brown rot development on Casselman plums. In contrast, the commercial ASPIRE™ (a.i. *Candida oleophila,* isolate I-182) and the semi-commercial biofungicide CIM (a.i., *Cryptococcus infirmo-miniatus*) did not reduce brown rot incidence.

Introduction.

Brown rot of stone fruit (*Prunus* spp.), caused by the ascomycete *Monilinia fructicola* (Wint.) Honey, is a serious disease in the California Central and Sacramento Valleys and routinely results in postharvest yield losses in excess of 30%. Currently, an estimated 100,000 hectares are under cultivation in the Sacramento and San Joaquin Valleys, producing annually some 800,000 metric tons of stone fruit (peaches, nectarines, apricots, plums, and prunes), valued at over 700 million dollars. The development of alternative strategies for chemical control of pre- and post-harvest brown rot of stone fruit could help the California *Prunus* industry to 1) monitor pathogen populations and predict brown rot, 2) reduce fungicide applications, 3) evaluate proper conditions in storage, and 4) establish quarantine measures based on species-specific detection method that affect directly imports of American stone fruit.

The pathogen *M. fructicola* causes infections, which become visible as blossom blight or decayed immature and mature fruit. In addition, the same organism can cause latent infections, which do not show any visible symptoms. There are two categories of latent infection by *M. fructicola* of stone fruit depending on the fruit developmental stage, when infection occurs, and the type of spore inoculum source. Long-term latent infections take place at an early stage of fruit development and result mainly from primary inoculum or inoculum produced in blighted blossoms (primary infections). Short-term latent infections take place at a later stage of fruit development and result mainly from typically secondary inoculum produced on thinned fruit, non-abscised aborted fruit, and infected injured green fruit. Fruit rot at harvest and postharvest is the result of survived long- or short-term latent infection. In our laboratory we are developing disease monitoring and diagnostic techniques that stone fruit growers can use to predict brown rot at harvest and postharvest and make "as-needed" fungicide applications. Specifically, this

project aims to evaluate alternative strategies for brown rot control that stone fruit growers can use in their orchards. The potential benefits of using monitoring brown rot by detecting latent infection could be: it could help growers (1) spray fungicides according to an as-needed programs instead of a calendar schedule, thus contributing to the overall reduction of fungicides used in stone fruit for brown rot control; (2) make decisions on which fruit to send first to the market and which to keep longer in cold storage based on the incidence of long- and short-term latent infection; (3) use less aggressive postharvest treatments for fruit that shows low level of latent infection; and (4) use, in general, more environmentally sound methods to manage a disease.

Assumptions of the study include (a) unconditional cooperation of participating growers during the time period of the study, and (b) environmental conditions would be favorable for the development of sufficient disease levels that could allow meaningful statistical comparisons. Significant progress towards these goals has been reported in our 1998 final report. The 1999 project is a continuation of the 1998 project.

Materials and Methods

Objective 1: To determine the relative importance of long- and short-term latent infection for pre- and post-harvest brown rot of stone fruits to help reduce sources of primary and secondary spore inoculum and predict disease at harvest and post-harvest:

(a) Detection of latent infection by overnight freezing-incubation technique (ONFIT). The collected fruit samples from the various orchards in 1998 were split in two halves, one half sample used for the ONFIT technique and the second half of each sample was frozen and it will be processed so that the DNA of natural *M. fructicola* infection is amplified and detected by the specific Mf2A and Mf2B primers and/or the genomic clones pMF73, pMF150, and pMF210. No additional samples were saved in 1999 because in preliminary experiments we found that the incidence of latent infection was <1%.

Because natural conditions were unfavorable for the development of latent infections, a study was initiated to determine conditions affecting latently infected fruit. These experiments were conducted in a prune orchard at Kearney Agric. Center from June through September 1999. Spore concentrations used in this field inoculation study were adjusted to 8,000, 16,000, and 24,000 conidia of *Monilinia fructicola* per ml. Five hundred ml spore suspension for each of the concentrations was made and 30 ml of each concentration were sprayed with a hand-held sprayer on branches of prune trees bearing 20 to 30 fruits. Immediately after inoculation, each branch was covered with a plastic bag and closed tightly to create high relative humidity around each inoculated branch. The three inoculation densities were applied at different branches of the same tree; non-inoculated control was used by spraying 30 ml sterile distilled water on a branch of a separate tree.

For each experiment, inoculations were conducted at about 20:00 SPT. Four wetness periods of 4, 8, 12, and 16 hours were accomplished by uncovering the plastic bags at 0:00 (midnight), 4:00, 8:00, and 12:00 SPT, respectively. Each of four trees was used as each of four wetness

periods. Three replicates for each inoculation density and wetness period and one replicate of water-inoculated control for each wetness period were used in each experiment. A data logger was used to automatically record hourly temperature during each experiment. Five experiments were conducted in July 1999 using similar methodology.

At the end of each wetness period and after removal of the cover plastic bag, inoculated branches were marked, and the fruit on each branch were kept on the trees until harvest. At commercial harvest, inoculated fruit showing no disease were harvested separately for each treatment of inoculum density and wetness period for each experiment. The ONFIT was used to determine the proportion of fruit infected with latent infections by *M. fructicola*. For each replicate of inoculation density and wetness period, fruit were surface sterilized in a chlorine solution (32 ml chlorine commercial sodium hypochlorite, 32 ml 95% ETOH, and 0.01 ml surfactant Tween-20 in 2 liters water) for 5 min. The fruit were then washed with sterile distilled water five times, and placed on a waxed wire screen in a plastic container (40 x 24 x 11 cm) with water at the bottom to increase humidity. The containers were placed in a freezer at -4^{0}C for 10 hours initially and then on a laboratory bench 23 ± 2^{0}C for 7 days. The proportion of fruit covered with sporulation of *M. fructicola* was recorded after 4 to 6 days.

Data analysis included analysis of variances, pair-wise comparisons of latently infected fruit between inoculum densities and wetness periods. Regression was conducted for each inoculum density so that the proportion of latently infected fruit was treated as the dependent variable and the wetness period and temperature as independent variables. Data combined from all experiments and inoculation densities were used for the overall regression analysis.

(b) Development of a nucleic acid-based system for detection of the latent infection. In winter 1998 and spring 1999, we spent a lot of time in setting up our molecular laboratory. At the same time we were able to complete all the instrumentation and technical training for the molecular procedures. With the exception of the mosquito lab, this is the only other molecular laboratory at the Kearney Agricultural Center. The developed specific primers, Mf2A and Mf2B, were used in early 1999 for the first time and were able to amplify and detect fungal DNA in stone fruit blossoms and stone fruit inoculated with *M. fructicola.* The next step is to peel the frozen fruit samples, extract the *M. fructicola* DNA, amplify it, and detect it by using these primers. However, post-doctoral associate Dr. Hong, the main player of the project, left for a permanent job in May 1999. Dr. Eric Boehm, an expert in molecular biology and population dynamics of pathogens, was hired and he continued and expanded the efforts initiated by Dr. Hong. Three students, Todd Esajian, Simon Gisler, and Robert Koja helped in preliminary ONFIT experiments and in other routine laboratory and field procedures.

Dr. Boehm used DNA from 300 random isolates of *Monilinia fructicola* in order to produce species-specific molecular diagnostic to detect and quantify *M. fructicola* from California stone fruits. Standard methodologies were used for DNA extractions and a partial library construction. The hybridization strategy used to identify species-specific, non-ribosomal repetitive clones in this study was threefold: (1) identification of endogenous, highly repeated plasmid clones using total DNA as the probe source; (2) exclusion of ribosomal clones using the entire 9.0Kb ribosomal repeat from *Nectria hematoccoca* as a heterologous probe; and (3) the determination

of specificity and sensitivity of the identified putative repeat clones. Under nonsaturating probe conditions and high stringency hybridization and wash temperatures, plasmid clones containing highly repeated sequences generated stronger signals than clones containing moderate to low or single copy sequences. This difference was the basis for the initial screen. Nonribosomal repetitive clones were then screened for specificity by dot blot hybridization to the 96 isolates of stone fruit fungal pathogens. These probes were shown to be specific to a worldwide collection of *M. fructicola* isolates, to the exclusion of other fungi commonly encountered from stone fruits surface, including the closely related *M. laxa.*

Objective 2: To epidemiologically and economically evaluate alternative strategies for chemical control of pre- and post-harvest brown rot of stone fruits. The strategies to be evaluated will include thinning of excess fruit at the post-pit-hardening stage and a) burying them underneath by disking or rototilling, and b) cover-spraying thinned fruit on the ground with a registered fungicide (vs. the conventional practice of leaving thinned fruit on the orchard floor):

In 1999, there was some difficulty in setting some of the above experiments. First of all, there was a discontinuity in the personnel involved in this project. Post-doctoral associate, Chuan Hong, has obtained a permanent research position and left in May. Field experiments were continued by Dr. Yong Luo who was hired in early July and molecular work by Dr. Eric Boehm who was hired in early August. Both these post-doctoral associates have been partially supported by the California Prune Board and a UC BioSTAR project, respectively. This discontinuity in personnel has created difficulties in the integrity of the project. In addition, the cooperating growers did not agree to do thinning of fruit at the post pit-hardening stage, and there was no orchard in our Center available to compare thinning of fruit at the pre-pit-hardening with thinning at the post-pit-hardening stage. Similarly, the cooperating growers did not allow us to set up the experiment for comparing rototilling thinned fruit vs. not rototilling as we have done in 1998. The main reasons for the lack of cooperation were that the majority of stone fruit growers now practice minimum or no tillage in their orchards.

Fortunately, one of the cooperating growers did thin fruit at a post-pit-hardening stage in two of his orchards, and we performed the experiment on cover-spraying thinned fruit on the orchard floor with iprodione (a registered fungicide) to compare the treatment with non-sprayed thinned fruit. In the first September Red nectarine orchard, a randomized block design included three plots of 11 x 12 trees per treatment. There were 2 treatments; the unsprayed control and the iprodione treated thinned fruit. Thinned fruit in the fungicide treatment were sprayed on 7 June 1999 with iprodione (Rovral®) at the label rate (1-lb a.i. iprodione), using a bicycle sprayer (mainly used to spray weeds in orchards). In the second August Red nectarine orchard, a randomized block design included three plots 11 x 11 trees per treatment. There were 2 treatments; the unsprayed control and the fungicide treated thinned fruit. In this orchard, thinned fruit were sprayed on 11 June 1999 also with Rovral® (1 lb. a.i. iprodione), using the same bicycle sprayer. In the September Red nectarine orchard, a random sample of 1,270 thinned fruit were recorded on the ground per plot each on 21 and 29 June, 21 July, 5 August, and 1 September 1999. Thinned fruit from a total of 35 trees were evaluated each time. The fruit were harvested on 6 September, and 300 fruit were recorded per tree in the 35 pre-flagged trees. In the August Red orchard, 1,150 thinned fruit on the floor per plot were evaluated each on 20 June, 2

and 20 July, and 4 and 20 August 1999. Thinned fruit from a total of 36 trees were evaluated each time. Fruit were harvested on 21 August 1999, and 300 fruit per tree were recorded in the 36 pre-flagged trees.

Objective 3: To expand on and select the best biological agent(s) for controlling post-harvest brown rot of stone fruits:

After Dr. Hong's departure from the Kearney Agricultural Center, an attempt to hire an individual to continue the biological control studies was not successful. A candidate with tremendous experience in biological control agreed to start on August 1, 1999. In July 1999, however, this candidate changed his mind and decided to go to New Zealand, his home country. This delayed the experiments for objective 3 of this proposal by at least 2 months.

Despite these difficulties, biological control experiments included selected species and isolates of *Trichoderma* spp., which were shown in 1998 to be very effective against *M. fructicola.* Also in these trials we included the compound 6PAP (6-pentyl-*2H*-pyran-2-one), a product produced by *Trichoderma viride* which was shown to be very effective against *B. cinerea.* The naturally derived 6PAP extracted from *T. viride* is approved by the United States Federal Drug Administration as a food additive.

Preparation of the pathogen. An isolate of *Monilinia fructicola* collected from a plum orchard and stored at the Kearney Agricultural Center was used in this study. *M. fructicola* from silica gel was cultured on Petri plates containing acidified potato-dextrose agar (APDA). The plates were incubated at 23 ± 2^{0}C for 5 days, and mycelium was transferred to new plates as needed. Three ml of sterile distilled water was poured in each plate to harvest conidia of *M. fructicola* by scraping the surface of the plate with a glass rod. In each biocontrol experiment, fresh spores of *M. fructicola* were harvested from sporulating plum fruit and were adjusted to 1000 spores/ml.

Preparation of biocontrol agents. Fungal and bacterial isolates used in this project were isolated from various stone fruits including mummies (Hong et al., 2000) and stored at Kearney Agricultural Center. The fungal and bacterial isolates from the agar slants were transferred into APDA plates. The concentration of each biological control isolate and biofungicides used in these experiments was adjusted to 10^8 spores or colony forming units (cfu)/ml.

Fruit inoculation and incubation. About 2,000 Casselman plum fruit were collected from an orchard at Kearney Agricultural Center in September 1999 and stored at 4^0 C. In each experiment, fruit were surface sterilized with a 400 ppm solution of chlorine made from household 5.25% sodium hypochlorite for 3 min. Fruit were placed on waxed wire screens in sterilized plastic containers (40 × 24 × 11 cm) with water at the bottom of the container to create a chamber with nearly 100% relative humidity. A sterile nail was used to make a wound (1 mm diameter and 2 mm depth) on the surface of each fruit. A drop of 10 to 20 µl of a spore suspension of a biological control agent was placed on each wound and for 6PAP a drop of 2.5 µl. For treatments of semi-commercial CIM and the commercial Aspire™ biofungicides, surface-sterilized fruit were dipped in a solution of either biofungicide (10^8 cfu/ml of water) for 1 min. Fruit were then inoculated with a drop of 10 to 20 µl of a *M. fructicola* spore suspension.

Fruit inoculated only with *M. fructicola* were used as the control treatment. There were three replications of 10 fruit each per treatment. The containers with the fruit were incubated at 25^0 C for 7 to 11 days when the diameter of the decay lesion was recorded after 4 days (for some), 7 days for all, and 11 days for some of the experiments. The size of lesions in mm^2 was then calculated

The biocontrol fungal and bacterial isolates used as treatments in this study and the figures of respective results are listed in Table 4.

Data analysis. Lesion area in each fruit from each treatment was used in data analysis. For each experiment, ANOVA was used to determine the variance of treatments and LSD was used to compare the effectiveness of biological control isolates.

Results

Objective 1: To determine the relative importance of long- and short-term latent infection for pre- and post-harvest brown rot of stone fruits to help reduce sources of primary and secondary spore inoculum and predict disease at harvest and post-harvest:

a) Detection of latent infection by overnight freezing-incubation technique (ONFIT).
No additional samples of stone fruit were collected in 1999 because in preliminary collections and ONFIT tests latent infections were nil to < 1%. Similarly, in samples collected from 18 prune orchards, the levels of brown rot ranged from 0 to 1% using ONFIT. However, in four sites the levels ranged from 2 to 6% (but this is believed to be misidentification of the brown rot pathogen). No fungicide treatments for fruit brown rot were recommended for any of the 22 sites based on the ONFIT fruit brown rot predictive model (Olson, *et al.,* 1999).

The effects of wetness period, inoculum density, and temperature on latently infected prune fruit by *M. fructicola* are described below:

None of the fruit in the water inoculated control at any wetness period in any experiment showed latent infection by *M. fructicola.* Results of overall ANOVA showed that the variances from experiment, wetness period, and inoculum density were all significant at $P<0.0001$, and all interactions among them were not significant (Table 1). Comparisons of spore inoculum concentrations demonstrated that the effect of spore density on secondary infection by *M. fructicola* was different among experiments (Table 2). Higher inoculum density (24,000 spores/ml) caused significantly more infected fruit than did lower inoculum density (8,000 spores/ml) for the different wetness periods and experiments (Table 2). When inoculum densities of 8,000 or 16,000 spores/ml were used, longer wetness period (*e.g.* 16 hours) led to more latent infections than did shorter wetness period (*e.g.* 4 hours) (Table 2). However, when inoculum density was 24,000 spores/ml, there was no difference between wetness periods in any of the five experiments (Table 2).

Regression analysis showed that when inoculum densities of 8,000 and 16,000 spores/ml were used, wetness period was a significant parameter ($P<0.01$) in affecting latent infection but temperature was not (Table 3). However, when inoculum density of 24,000 spores/ml was used, the

wetness period was not significant while temperature became more significant at $P<0.05$ (Table 3). The overall regression from combination of inoculum densities showed that wetness period and inoculum density were both significant at $P<0.0001$, and temperature was significant at $P<0.05$ (Table 3).

b) Development of a nucleic acid-based system for detection of the latent infection.
In addition to the two PCR primers, Mf2A and Mf2B, we have identified three high copy, genomic clones (pMF73, pMF150 and pMF210) from a partial Sau3AI genomic library that are species-specific to a world-wide collection of *M. fructicola* isolates, to the exclusion of other fungi commonly encountered from the stone fruit surface, including the closely related *M. laxa*. These three high copy clones are sensitive enough to detect less than 50 pg of fungal DNA in dot blot hybridizations. From reconstruction experiments, clone pMF73 comprises roughly 2.0% of the genome and exists in approximately 2500 copies per haploid genome. Two of these clones (pMF73 and pMF150) cross-hybridize and are extra-chromosomal in origin. The arrangement of the extra-chromosomal clones is conserved throughout a geographically diverse assemblage of *M. fructicola* isolates (CA, MI, GA, OR, and Australia). The third clone (pMF210) migrates with uncut DNA and is nuclear in origin. All three clones are tandemly arranged in the genome, as opposed to interspersed, and represent multiple copies of variable number of tandem repeat sequences. These genomic clones will be used to detect *M. fructicola* in symptomless stone fruit blossoms and fruits.

Objective 2: To epidemiologically and economically evaluate alternative strategies for chemical control of pre- and post-harvest brown rot of stone fruits:

None of the thinned fruit in either treatment (sprayed with iprodione or not sprayed) showed any sporulation by *M. fructicola.* Neither any brown rot disease developed in the field at harvest or after postharvest incubation. In 1999 the conditions were very unfavorable for the development of latent infections, sporulation of *M. fructicola* on thinned fruit, infection of mature fruit, and development of postharvest brown rot in the orchards where these experiments took place.

Objective 3: To expand on and select the best biological agent(s) for controlling post-harvest brown rot of stone fruits:

The results are presented by experiment as follows:
Experiment 1. In this experiment, the anti-fungal metabolite 6PAP (6-pentyl-*2H*-pyran-2-one) produced by *Trichoderma* species was used as one of the treatments. Differences in lesion size caused by *M. fructicola* on fruit among the three treatments were significant (Figure 1). Treatments of fruit wounds with 2.5 µl 6PAP significantly reduced the size of lesions caused by *M. fructicola.* In a similar way, a spore suspension of *Trichoderma* itself also significantly reduced brown rot lesion size. After 6 and 11 days, the reduction caused by the 6PAP (for both 10 and 20 µl concentration of *M. fructicola*) was significantly greater than that caused by the spore suspension of *Trichoderma* species (Figure 1).

Experiment 2. In this experiment the 6PAP and the spore suspension of *Trichoderma* treatments were compared with a semi-commercial (CIM= active ingredient *Cryptococcus infirmo-*

miniatus) and a commercial (Aspire™ = active ingredient *Candida oleophila* isolate I-182) biofungicides. Only the 6PAP and *Trichoderma* treatments were effective in reducing brown rot lesions in this experiment (Figure 2). Again, 6PAP was more effective than the spore suspension of *Trichoderma* species. However, the biofungicides showed no effect in controlling brown rot (Figure 2).

Experiment 3. In this experiment, the bio-fungicides CIM and Aspire™ were compared with an untreated control treatment. Because disease developed fast in all treatments, lesions were measured only 4 days after inoculation. CIM and Aspire™ not only did not reduce lesion size by *M. fructicola*, but instead it resulted in a slight and significant increase of lesion size in comparison with the lesions of the control treatment (Figure 3). Interestingly, the commercial bio-fungicide Aspire™ resulted in significantly larger lesions than the semi-commercial CIM. Therefore, these bio-fungicides proved to be ineffective against controlling brown rot in stone fruits, thus confirming results of Experiment 2.

Experiment 4. In this experiment, other fungi isolated from various stone fruits were used at concentrations described above. All three biological control agents, *Fusarium*, *Paecilomyces,* and *Penicillium* reduced lesion size significantly (Figure 4). However, treatment of fruit with *Penicillium* was more effective in reducing severity of disease in comparison with the *Fusarium* or *Paecilomyces* treatments (Figure 4).

Experiment 5. In this experiment, another *Trichoderma* isolate and a *Bacillus subtilis* isolate were compared with the untreated but inoculated *M. fructicola* control treatment. These two biological treatments were the most effective among all treatments tested because they totally prevented brown rot lesion development 7 days after inoculation (Figure 5).

Experiment 6. In this experiment, spore suspensions of each a *Gloeosporium* and a yeast treatment were compared with the untreated but inoculated *M. fructicola* control treatment. Both these treatments significantly reduced lesion size 7 days after inoculation and incubation at 25^0 C (Figure 6).

Discussion

Because of unfavorable conditions for development of latent infections by *M. fructicola* in 1999, it was not possible to validate the ONFIT procedure, which provided significant correlation of latent infections of stone fruit with decay at harvest and postharvest in during 1994 to 1996 (Michailides et al., 2000). In fact, we provided the protocol of the technique for a study done in peaches in Georgia and we had the same success in using latent infection as a predictor of brown rot risk (Emery et al., 2000). We expect that in years when conditions are favorable for development of latent infection these infections can be used to predict brown rot risk at harvest and in postharvest storage. Using inoculation of green prunes, we identified that wetness duration, and inoculum concentration as the main factors affecting the incidence of latent infection. Therefore, the lack of latent infections in 1999 stone fruit could be explained by the short wetness periods (lack of late rains) and absence of secondary inoculum produced in thinned fruit.

We were successful in developing two very species-specific primers (MF2A and MF2B) that amplified DNA from *M. fructicola* as well as from stone fruit blossoms and fruit inoculated with the pathogen. We are at one step closer to make detection of latent infections in fruit naturally infected by *M. fructicola.* More importantly, we have identified three high copy, genomic clones (pMF73, pMF150, and pMF210) that are specific to a world-wide collection of *M. fructicola* isolates, to the exclusion of other fungi commonly encountered from stone fruit surface, including the closely related *M. laxa.* These high copy clones are sensitive enough to detect between 10-50 pg of fungal DNA in dot blot hybridizations. Using these clones we can not only detect but also quantify *M. fructicola* from California stone fruits (Boehm et al., 2000). Analyses of fruit with suspected latent infections of *M. fructicola* collected from 29 orchards in 1998 using the PCR primers and the specific genomic clones are in progress. A UC BioSTAR project is supporting the continuation of this aspect of research.

In 1998 experiments, spraying iprodione on thinned fruit on the orchard floor significantly reduced sporulation by *M. fructicola* (Michailides, 1998). In contrast, the 1999 results of the same experiment in two commercial orchards were negative because of unfavorable conditions for the development of sporulation on any thinned fruit (treated and non-treated). Also in 1998 we showed that rototilling thinned fruit could eliminate most of the thinned fruit on the orchard floor and prevent sporulation (Michailides, 1998). Thinned fruit was shown to be a significant source of secondary inoculum for the brown rot pathogen (Hong et al., 1998). It was unfortunate that we were not able to repeat some of these types of experiments in 1999 because of reasons explained in the "Materials and Methods" section of this report.

Biological agents isolated from various stone fruits (Hong et al., 2000) that showed effective biocontrol against brown rot in previous years Hong et al., 1998) were re-tested using Casselman plums. In addition to *Trichoderma* spp., species of *Fusarium, Paecilomyces, Penicillium, Gloeosporium, Bacillus,* and a yeast isolate were tested in 1999. Most importantly, the metabolite 6PAP produced by a *Trichoderma* species was very effective in reducing severity of brown rot. The semi-commercial CIM (a. i., *Cryptococcus infirmo-miniatus*) and the commercial Aspire® (a.i., *Candida oleophila*) bio-fungicides which have been reported to be effective by other researchers were shown to be ineffective against brown rot even after applying them in fruit wounds before inoculation with *Monilinia fructicola.* The general conclusion from these trials was that the most effective biocontrol treatments were a *Trichoderma* and a *Bacillus subtilis* isolates, which totally prevented brown rot in Casselman plums.

Conclusions:

1. Two very specific PCR primers, Mf2A and Mf2B, have been developed, amplified, and detected DNA of *Monilinia fructicola* in stone fruit blossoms and inoculated fruit. Analyses of stone fruit samples collected from 29 orchards in 1998 are in progress.
2. In addition, three genomic clones (pMF73, pMF150, and pMF210) have been developed, amplified, detected, and quantified DNA of a worldwide collection of *M. fructicola.* Tests with these clones using naturally infected blossoms and fruits are in progress.

3. In contrast to 1998 results, conditions for brown rot development were very unfavorable in 1999. No conclusion could be made from two orchard experiments where thinned fruit on the floor were sprayed with iprodione because sporulation did not develop in any of the fruit in either treatment.
4. Results from the biological control experiments showed that the natural product 6PAP produced by *Trichoderma* spp. was very effective in entirely preventing brown rot infection of Casselman plums 6 days after inoculation while the control was 100% infected. The most effective biocontrol treatments were *Trichoderma* and Bacillus *subtilis* isolates, which totally prevented brown rot.

Literature Cited and List of Publications Produced:

* Boehm, E. W., Ma, Z., and Michailides, T. J. 2000. Development of a species-specific molecular diagnostic to detect and quantify *Monilinia fructicola* from California stone fruits. (Abstr.). Phytopathology 90: (in press).

Emery, K.M., Michailides, T.J., and Schrem, H. 2000. Incidence of latent infection of immature peach fruit by *Monilinia fructicola* and relationship to brown rot development in Georgia. Plant Disease 84 (in press).

* Hong, C. X., Holtz, B. A, Morgan, D. P., and Michailides, T. J. 1998. Significance of thinned fruit as a source of the secondary inoculum of *Monilinia fructicola* in California nectarine orchards. *Plant Disease* 81:519-524.

* Hong, C. X., Michailides, T. J., and Holtz, B. A. 1998. Effects of wounding, inoculum density, and biological control agent on post-harvest brown rot of stone fruits. *Plant Disease* 82:1210-1216.

* Hong, C.X., Michailides, T. J., and B. A. Holtz. 2000. Mycoflora of stone fruit in California orchards. Plant Disease 84: (in press).

* Michailides, T. J. 1998. Development of alternatives strategies for chemical control of preharvest and post-harvest brown rot of stone fruits. DPR Progress Report 1998. Sacramentro, 16 pp.

* Michailides, T. J., Morgan, D.P., and Felts, D. 2000. Detection and significance of latent infection of *Monilinia fructicola* in California stone fruits. (Abstr.). Phytopathology 90: (in press).

Olson, W. et al. 1999. Environmentally sound prune Systems (ESPS). Pages 173-194 in: Prune Research Reports and Index of prune Research. California Prune Board, Pleasanton.

* Indicates publications produced.

APPENDICES

Table 1. Results of ANOVA of proportion of latently-infected prune fruit infected by *Monilinia fructicola*. Fruit on trees were inoculated with different inoculum densities, and kept under different wetness periods. Three replicates were used for each combination of inoculum density and wetness period in each experiment. Five experiments were conducted.

Source*	DF	F Value	Pr > F
exp	4	9.46	<.0001
wtp	3	8.13	<.0001
ind	2	22.34	<.0001
exp x wtp	12	1.53	0.1257
exp x ind	8	1.89	0.0687
wtp x ind	6	0.73	0.6228
exp x wtp x ind	23	1.51	0.0822

* exp= experiment; wtp= wetness period; ind= inoculum density.

Table 2. Comparisons of proportions of latently-infected prune fruit by *Minilinia fructicola* between wetness periods and inoculum. Each value was the mean of three replicates.

Experiment	Inoculum density (spores/ml)	Wetness period (hrs.) 4	8	12	16
1	8,000	20.3 A[1] a[1]	- [2]	13.5 B a	30.2 A a
	16,000	30.5 A ab	8.1 A b	48.6 A a	14.9 A ab
	24,000	33.6 A a	25.5 A a	30.7 A a	28.7 A a
2	8,000	0 A b	8.4 A b	12.0 A b	29.7 A a
	16,000	0 A c	20.1 A b	17.2 A b	34.7 A a
	24,000	12.9 A a	20.6 A a	33.3 A a	33.1 A a
3	8,000	20.4 A a	12.7 A a	-	14.7 B a
	16,000	5.2 A c	24.8 A b	30.5 A b	51.6 A a
	24,000	38.2 A a	45.5 A a	41.5 A a	49.9 A a
4	8,000	12.9 B a	28.5 A a	34.2 A a	34.0 A a
	16,000	20.8 B b	35.8 A ab	43.2 A ab	47.2 A a
	24,000	38.4 A a	64.4 A a	58.3 A a	67.5 A a
5	8,000	4.1 B b	12.1 A ab	26.3 A a	24.1 A a
	16,000	23.9 A a	30.3 A a	23.5 A a	26.6 A a
	24,000	33.9 A a	24.9 A a	25.6 A a	31.6 A a

[1] Comparisons between inoculum densities for each wetness period and between wetness period for each inoculum density are represented by upper case and lower case letters, respectively. Values followed by a common letter are not significantly different using LSD test at $P \leq 0.05$.
[2] Missing values.

Table 3. Results of regression analysis on proportions of latently-infected prune fruit by the pathogen *Monilinia fructicola* vs temperature and wetness period for different inoculum densities and overall regression after combining inoculum densities.

	Inoculum density								
	8,000 spores/ml			16,000 spores/ml			24,000 spores/ml		
Variable	DF	Parameter estimate	t	Pr>|t|	Parameter Estimate	t	Pr>|t|	Parameter Estimate	t	Pr>|t|
Intercept	1	3.55	0.24	0.8137	26.97	1.87	0.0670	63.04	3.71	0.0005
T	1	0.11	0.18	0.8566	-0.74	-1.23	0.2258	-1.58	-2.14	0.0370
W	1	1.18	2.76	0.0080	1.61	3.55	0.0008	0.64	1.21	0.2334

Overall regression of combined inoculum densities:

Variable[1]	DF	Parameter estimate	t Value	Pr > |t|
Intercept	1	14.48181	1.55	0.1230
I	1	1.11680	5.91	<.0001
T	1	-0.78711	-2.04	0.0426
W	1	1.13342	4.14	<.0001

[1] T= temperature; W= wetness period; I= inoculum density.

Table 4. Summary of biocontrol experiments against *Monilinia fructicola*, corresponding tested agents, and results.

Experiment	Treatment	Collection reference number	Fruit used	Results presentation
Experiment 1	Control (*M. fructicola*) *Trichoderma* species 6PAP (10ul) 6PAP (20ul)		Plum	Figure 1
Experiment 2	Control (*M. fructicola*) 6PAP CIM *Trichoderma* species Aspire®		Plum	Figure 2
Experiment 3	Control (*M. fructicola*) CIM Aspire®		Plum	Figure 3
Experiment 4	Control (*M. fructicola*) *Paecilomyces* species *Fusarium* *Penicillium* species	 *2* *3* *1*	Plum	Figure 4
Experiment 5	Control (*M. fructicola*) BCA *Bacillus* *Trichoderma* species	 *19* 24	Plum	Figure 5
Experiment 6	Control (*M.* fructicola) *Gloeosporium* species Yeast	 26 17	Plum	Figure 6

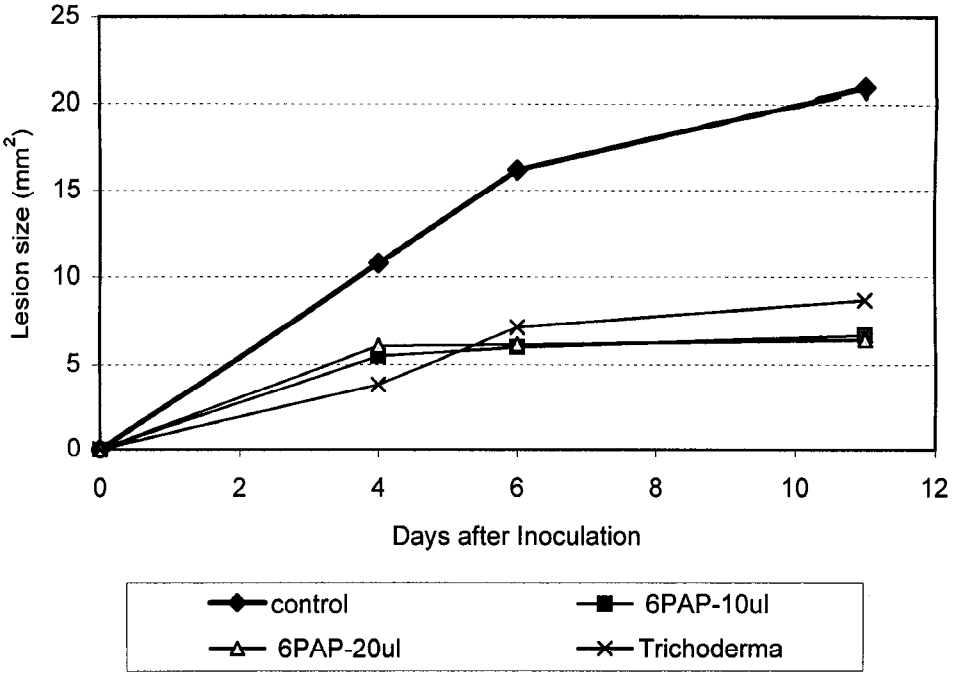

Figure 1. Effects of 6PAP and spore suspension of a *Trichoderma* isolate in controlling brown rot caused by *Monilinia fructicola* on Casselman plums. [6PAP-10 ul and 6PAP-20 ul means that fruit were inoculated with 10 and 20 ul of *M. fructicola* spore suspension per wound, respectively.]

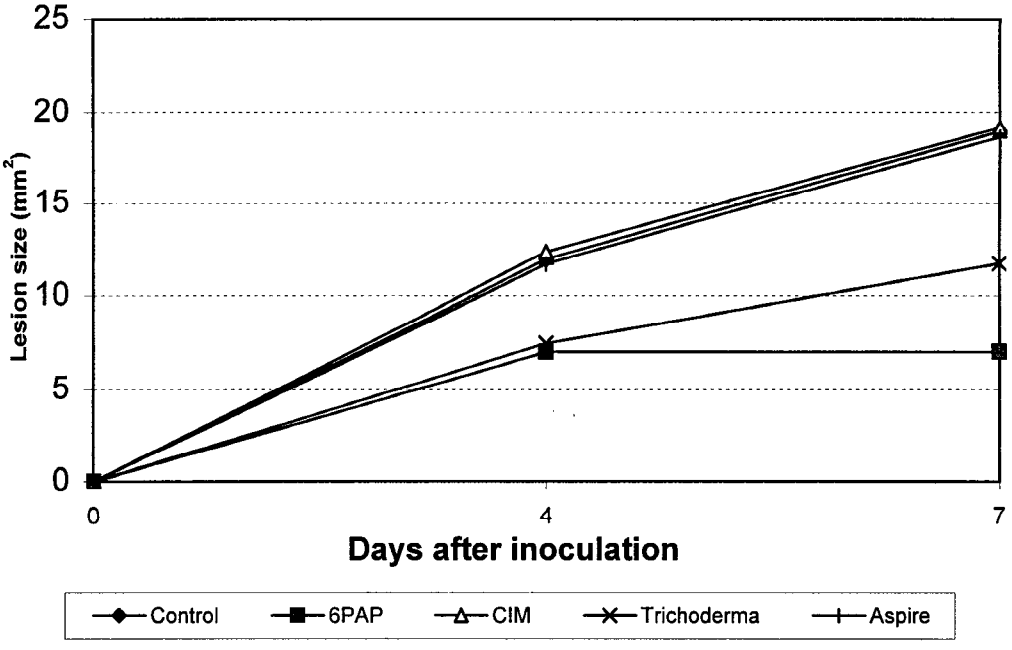

Figure 2. Effects of 6PAP, spore suspension of a *Trichoderma* isolate, and two bio-fungicides in controlling brown rot on Casselman plums caused by *Monilinia fructicola*.

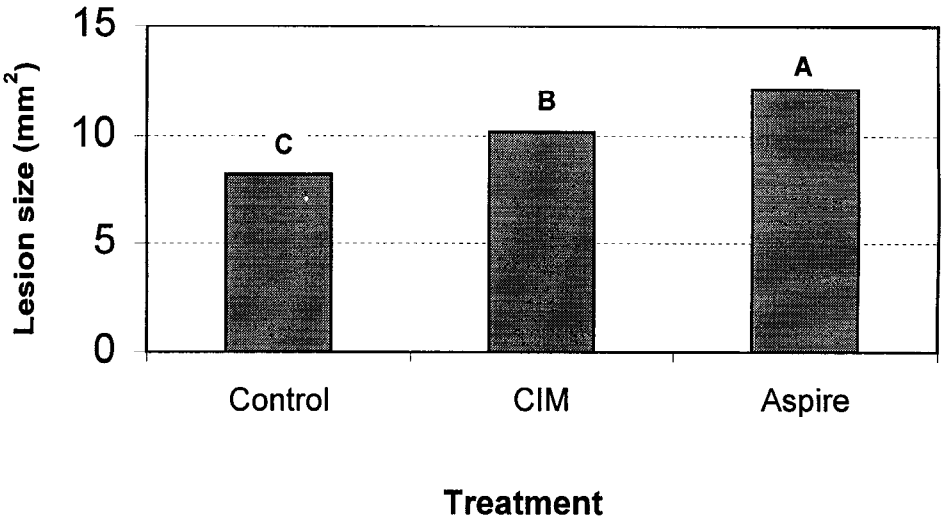

Figure 3. Effects of two biofungicides (CIM = a.i. *Cryptococcus infirmo-miniatus* and Aspire™ = a.i. *Candida oleophila*) on brown rot of Casselman plums caused by *Monilinia fructicola*.

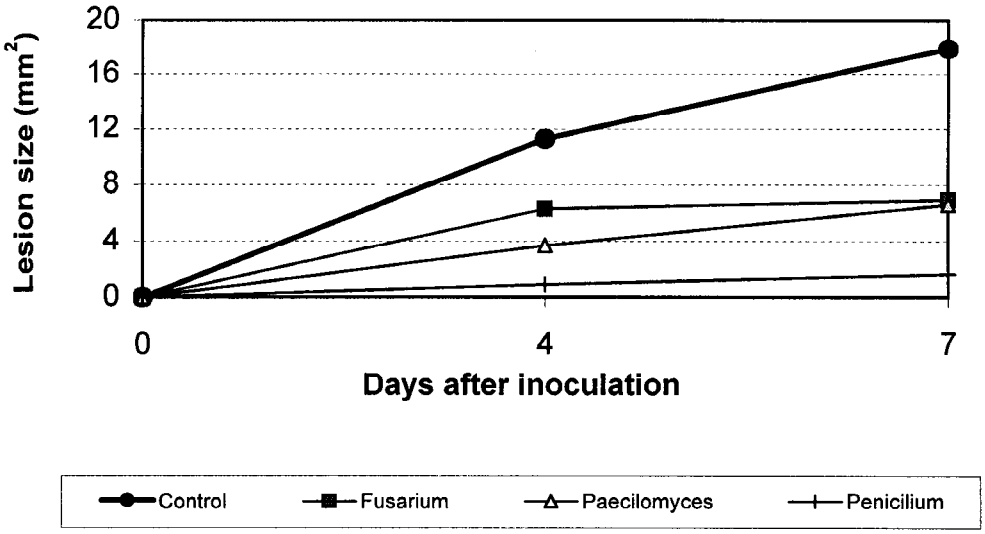

Figure 4. Effects of various biological control agents on brown rot of Casselman plums caused by *Monilinia fructicola*.

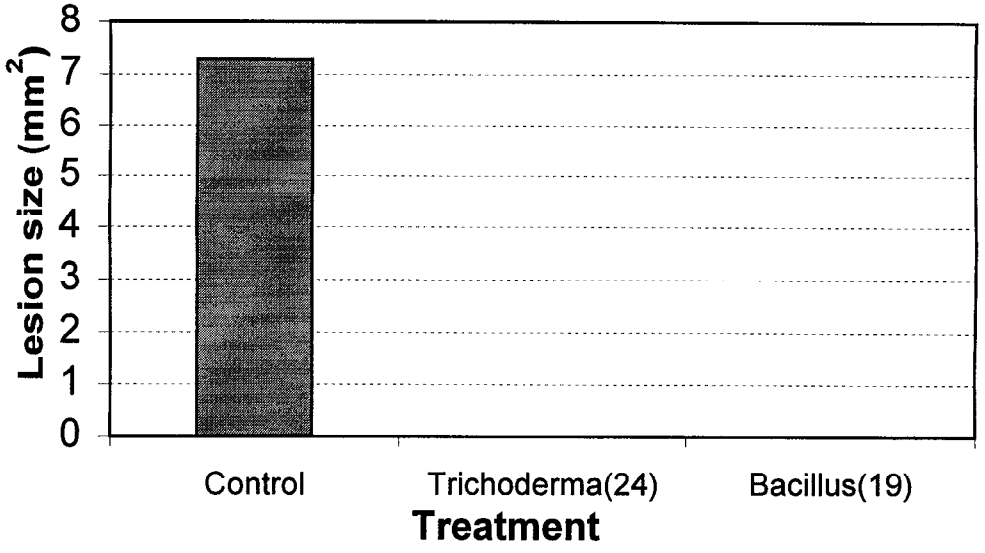

Figure. 5. Effects of an isolate of *Trichoderam* and a *Bacillus* species on brown rot of casselman plums caused by *Monilinia fructicola.*

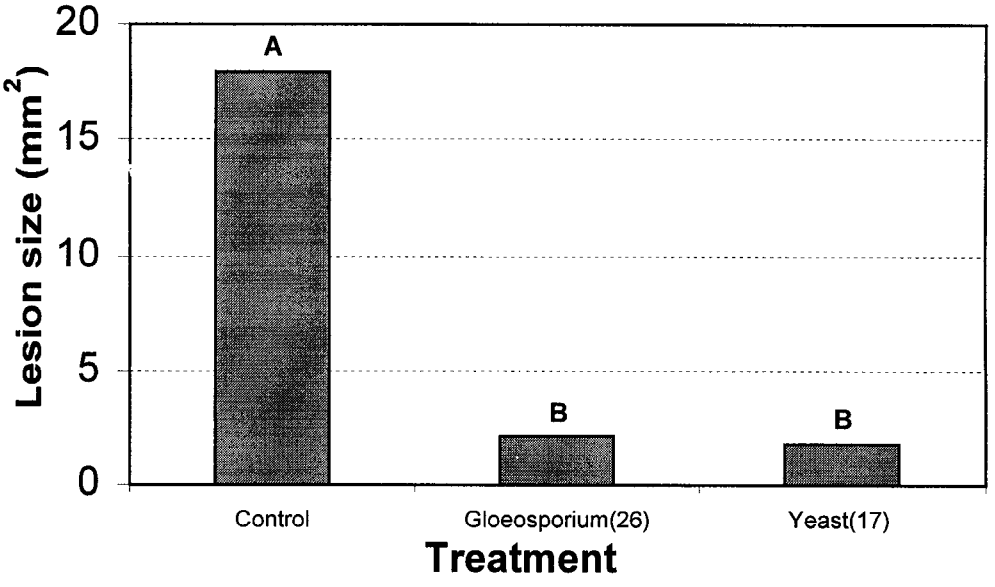

Fig. 6. Effects of *Gloeosporium* and a yeast isolate on brown rot of Casselman plums caused by *Monilinia fructicola* .